GENE INVOLVED IN THE BIOSYNTHESIS OF CAROTENOID AND MARINE MICROORGANISM, PARACOCCUS HAEUNDAENSIS, PRODUCING THE CAROTENOID

FIELD OF THE INVENTION

The present invention relates to genes involved in the biosynthesis of carotenoid and a marine microorganism producing the carotenoid.

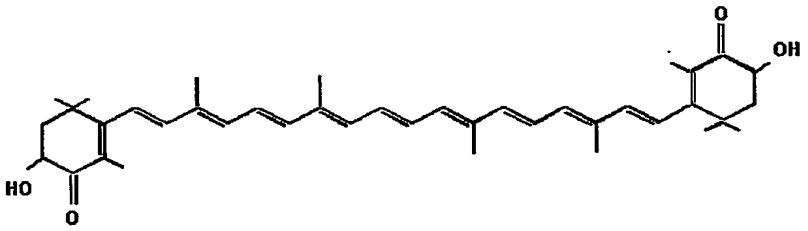
BACKGROUND

Carotenoid, a C40 isoprenoid compound having an anti-oxidant activity, means a group of pigment that is widely distributed in the nature. More than 600 kinds of carotenoids have been known so far, and they are all in different forms. The color of carotenoid varies from its molecular structure; that is whether it is yellow, red, scarlet or orange is decided upon the molecular structure of carotenoid. The examples of carotenoids are β-carotene (an orange pigment included in a carrot), licopene (a red pigment included in a tomato), fucoxanthin (a yellowish brown or a brown pigment included in marine

plants), etc. As a precursor of vitamin A in human, carotenoid has activities of preventing oxidation, scavenging harmful oxygen, inhibiting the proliferation of cancer cells, and preventing the development of a cancer. It suggests that it has preventive effect on cardiovascular diseases, cancers and other adult diseases. It has been disclosed recently that carotenoid enhances immunity as being exposed on UV, so that it reduces skin damages by UV or inhibits the production of melanin. Since then, carotenoid came into a spotlight as a cosmetic material in Europe and in the U.S.A. Carotenoid is now in use as a health food ingredient (nutritional supplement), a pharmaceutical composition and a food-coloring agent, or as a pigment for animal feeds.

Among many carotenoids, astaxanthine (3, 3'-dihydroxy-β ,β -carotene-4, 4'-dione) having the structure of below <Chemical Formula 1> is a scarlet or light orange color pigment produced in nature.

<Chemical Formula 1>



Astaxanthine is mostly included in tissues of marine animals such as shrimps, red seabreams, salmons and lobsters, etc (Fujita *et al.*, *Nippon Suisan Gakkaishi.*, 49: 1855-1869, 1983; Johnson, E. A., *Crit. Rev. Biotechnol.*, 11: 297-326, 1991; Nelis *et al.*, *J. Appl. Bacteriol.*, 70: 181-191, 1991). Astaxanthine not only inhibits the reactions of active oxygen to destroy DNA, proteins and lipids in cells during aerobic metabolism, to cause aging in cells and tissues, and to induce a cancer but also suppresses the generation of hydroxy or peroxy radicals (hydroxy or peroxy radicals (Palozza *et al.*, *Arch. Biochem. Biophys.*, 297: 291-295, 1992; Shimidzu *et al.*, *Fish Sci.*, 62: 134-137, 1996). In addition, astaxanthine has been known to have immune modulatory activity and cardioprotective effect (Jyonuchi *et al.*, *Nutr. Cancer.*, 19: 269-280, 1993). In particular, an antioxidant activity of

astaxanthine is 10 times as high as that of other carotenoids and 100 times as high as that of α-tocopherol. However, toxicity of astaxanthine has not been reported as of today. Astaxanthine has been widely used for the treatment and the prevention of various diseases including neurodegenerative diseases, cancers, immune disorders, cardiovascular diseases, etc, and studies are still going on further (Beal, H. F., *The Neuroscientist*, 3: 21-27, 1991; Chew *et al.*, *Anticancer Res.*, 19: 1849-1853, 1999; Murillo E., *Arch. Latinoam. Nutr.*, 42: 409-413, 1992). Astaxanthine is also being industrially used as a coloring agent and has been registered as a food additive in the name of 'Phaffia color' in Korea. The consumption of astaxanthine increases over 15% every year, suggesting the importance of astaxanthine.

A method for chemical synthesis of astaxanthine has been recently developed by a company (Hoffman-LaRoche, Switzerland). However, a synthesized astaxanthine showed lower *in vivo* absorption and weaker stability as a food additive than a natural astaxanthine, so that the use of the synthesized astaxanthine was allowed just in

some of European countries. Thus, a way to synthesize a natural astaxanthine is in strong demand and especially a way to produce astaxanthine using a microorganism producing astaxanthine becomes the focus of industrial interest. *Phaffia rhodoxyma* (Miller *et al.*, *Int. J. Syst. Bacteriol.*, 48: 529-536, 1976), a kind of yeast, *Haematococcus pluvialis* (Bubrick, *Bioresour Technol.*, 38: 237-239, 1991), a kind of *Chlorophyta*, Gram-positive *Brevibacterium* 103 (Lizuka & Nishimura, *J. Gen. Appl. Microbiol.*, 15: 127-134, 1969), Gram-negative *Agrobacterium aurantiacum* (Yokoyama *et al.*, *Biosci. Biotechnol. Biochem.*, 58: 1842-1844, 1994), *Paracoccus marcusii* (Harker *et al.*, *Int. J. Syst. Bacteriol.*, 48: 543-548, 1998) and *Paracoccus carotinifaciens* (Tsubokura *et al.*, *Int. J. Syst. Bacteriol.*, 49: 277-282, 1999) are the examples of the microorganisms producing astaxanthine.

Studies have been focused on a gene coding an enzyme involved in biosynthesis of carotenoid for the past 6 years. As a result, a number of genes involved in biosynthesis of carotenoid were cloned from various microorganisms and functions of them

were also examined (Armstrong, G. A., *J. Bacteriol.*, 176: 4795-4802, 1994; Sandmann, G., *Eur. J. Biochem.*, 223: 7-24, 1994; Wieland, B., *J. Bacteriol.*, 176: 7719-7726, 1994). The pathway of carotenoid biosynthesis is derived from FPP (farnesyl pyrophosphate), which is an intermediate product of general isoprenoid synthesis pathway. As seen in FIG. 8, FPP and IPP (isopentenyl pyrophosphate) turn into GGPP (geranylgeranyl pyrophosphate) by geranylgeranyl pyrophosphate synthase encoded by *crtE*. Then, GGPP turns into β -carotene by the reactions of phytoene synthase encoded by *crtB*, phytoene desaturase encoded by *crtI* and lycopene cyclase encoded by *crtY*. β -carotene changes into astaxanthine finally by the reactions of β -carotene ketolase encoded by *crtW* and β -carotene hydroxylase encoded by *crtZ*.

Nucleotide sequences, an organization and characteristics of *crt* gene (carotenogenic gene) involved in biosynthesis of carotenoid have been investigated in *Rhodobacter capsulatus* (Armstrong *et al.*, *Mol. Gen. Genet.*, 216: 254-268, 1989), *Erwinia herbicola* (Sandimann *et al.*, *FEMS Microbiol. Lett.*, 71: 77-82, 1990; Hundle *et al.*, *Photochem. Photobiol.*, 54: 89-93, 1991) and

Ervinia uredovora (Misawa *et al.*, *J. Bacteriol.*, 172: 6704-6712, 1990). Besides, *crt* gene involved in biosynthesis of carotenoid, which is composed of *crtB*, *crtI*, *crtY*, *crtW* and *crtZ*, has been isolated from *Agrobacterium aurantiacum*, a marine microorganism (Norihiko *et al.*, *J. Bacteriol.*, 177(22): 6575-6584, 1995). Another report has been made on three genes (*crtB*, *crtI* and *crtY*) coding enzymes catalyzing reactions from GGPP to β-carotene and *Phaffia rhodozyma* where those three genes are inserted (WO 97/23633).

Thus, the present inventors isolated and identified a novel *Paracoccus* genus microorganism producing astaxanthine and studied further to separate a gene involved in biosynthesis of carotenoid from the microorganism. As a result, the present inventors successfully cloned *crtE*, *crtB*, *crtI*, *crtY*, *crtW* and *crtZ* genes and *crt* gene containing all of the above genes as well, and then the inventors examined nucleotide sequences of them, too. The present inventors completed this invention by confirming that carotenoid could be produced by using the *crt* gene in microorganisms that were not able to produce

carotenoid.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a *Paracoccus haeundaensis* producing astaxanthine.

It is also an object of this invention to provide a protein needed in biosynthesis of carotenoid and a gene having nucleotide sequences selected from a group consisting of nucleotide sequences represented each by SEQ. ID. No 5, No 7, No 9, No 11, No 13 and No 15.

It is a further object of this invention to provide a gene involved in biosynthesis of carotenoid containing the above gene. In particular, the present invention provides a *crt* gene having nucleotide sequences represented by SEQ. ID. No 4.

It is another object of this invention to provide a recombinant vector containing the gene involved in biosynthesis of carotenoid above.

It is another object of this invention to provide a method for producing carotenoid using the above gene involved in biosynthesis of carotenoid.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to achieve the above object, the present invention provides a *Paracoccus haeundaensis* producing astaxanthine.

The present invention also provides a protein needed in biosynthesis of carotenoid and a gene having nucleotide sequences selected from a group consisting of nucleotide sequences each represented by SEQ. ID. No 5, No 7, No 9, No 11, No 13 and No 15.

The present invention further provides a gene involved in biosynthesis of carotenoid containing the above gene. In particular, the present invention provides *crt* gene having nucleotide sequences represented by SEQ. ID. No 4.

The present invention also provides a recombinant vector containing the gene involved in biosynthesis of carotenoid above.

The present invention further provides a method for producing carotenoid using the above gene involved in biosynthesis of carotenoid.

Hereinafter, the present invention is

described in detail.

The present invention provides a *Paracoccus haeundaensis* producing astaxanthine.

A microorganism was isolated from seawater sample taken from Haeundae shore in Busan, Korea as a strain having orange or red color. After investigating characteristics of the strain, it was confirmed that the strain was a rod type Gram-negative bacterium having non-motility and did not form spores (see FIG. 1). The cell size was 0.3-0.7 ㎛ in diameter and 0.8-2.5 ㎛ in length. The colony had orange (scarlet) color. The optimum growth temperature of the strain was 25℃, and the strain was never growing under 10℃ or over 40℃ (see FIG. 2). The optimum NaCl concentration for the growth was 1-6%(w/w) and the strain was not growing at all with over 8% (see FIG. 4). The optimum growth pH of the strain was 8 (see FIG. 3).

The strain of the present invention used only D-arabinose and galactose as a carbon source and an energy source for the growth. Neither pentoses, hexoses, sugar alcohols, ogligosaccharides nor other amino acids were used. Starch hydrolysis, cytochrome oxidase and catalase reactions were all positive. But, urease reaction was negative. The

strain could not produce indole from tryptophane, but took advantage of citric acid in deed, confirmed by citric acid test. Denitrification test was also performed. As a result, the strain reduced nitrate to nitrite, but did not reduce nitrite to N_2 gas. Besides, the strain did not ferment glucose. The strain of the present invention was also confirmed to be aerobic. DNA G+C composition of the strain was 66.9 mol%. The major non-hydroxyl fatty acid was unsaturated C18:1, and the major hydroxyl fatty acid was C10:0 (3-OH).

In general, a *Paracoccus* genus microorganism is an oxidase and catalase positive, gram-negative bacterium and belongs to α-3-subclass of *Proteobacteria* phylogenetically. Other characteristics of the microorganism were also investigated (see Table 2). As a result, it was confirmed that the strain of the present invention belonged to *Proteobacteria*.

In order to identify the strain of the present invention more concretely, the inventors examined sequence of 16S rDNA. As a result, the nucleotide sequence of 16S rDNA of the strain of

the invention had a high homology with those of *Paracoccus marcusii* and *Paracoccus carotinifaciens*. Nevertheless, the strain of the present invention showed different characteristics, comparing to other *Paracoccus* genus microorganisms including the two above (see Table 4 and Table 5). Therefore, the present inventors confirmed that the strain of the invention was a novel *Paracoccus* genus microorganism and named it '*Paracoccus haeundaensis*'.

The present inventors investigated if carotenoid was generated in the *Paracoccus haeundaensis*, resulting in the confirmation that the strain of the present invention produced β - carotene and astaxanthine (see FIG. 7). The present inventors deposited the *Paracoccus haeundaensis* of the present invention at KCCM (Korean Culture Center of Microorganisms) on January 24, 2003 (Accession No: KCCM - 10460).

Paracoccus haeundaensis of the present invention can be effectively used for the production of carotenoid, especially astaxanthine. A method to produce carotenoid using a microorganism of the present invention includes

the steps of culturing *Paracoccus haeundaensis* in a proper medium and collecting astaxanthine from the culture solution. Particularly, the strain of the present invention was cultured in PPES-II medium at 25℃ for 4 days, which was the primary culture, then cells were collected from the culture solution. Organic solvent was added to the cells, which were cultured at 4℃ for overnight, resulting in the elution of astaxanthine. In addition to the above PPES-II medium, LB3 medium (LB medium complemented with 3% NaCl) can be used as a medium for the culture of the strain. It is also preferable to add galactose to a medium in order to induce satisfactory growth of the strain and mass-produce astaxanthine. Methanol, acetone or ethyl ether can be used as an organic solvent used for the purification of astaxanthine from the cultured cells, and methanol is more preferably used. The collection of astaxanthine from the cultured cells can be performed using HPLC (high performance liquid chromatography) or TLC (thin-layer chromatography) by the conventional method known to the people in this field.

The present invention also provides a protein required for biosynthesis of carotenoid and a gene having nucleotide sequences selected from a group consisting of sequences each represented by SEQ. ID. No 5, No 7, No 9, No 11, No 13 and No 15.

The gene above means a gene producing astaxanthine purified from *Paracoccus haeundaensis* (Accession No: KCCM-10460).

It is preferred for a gene coding a protein required for biosynthesis of carotenoid of the present invention to have a sequence selected from a group consisting of sequences each represented by SEQ. ID. No 5, No 7, No 9, No 11, No 13 and No 15. Each protein coded by each gene above has amino acid sequence represented by SEQ. ID. No 6, No 8, No 10, No 12, No 14 and No 16, respectively. 6 genes of the present invention and proteins coded by the same are shown in the below Table 1.

<Table 1>

Gene	Gene name	Protein	Amino acid sequence
SEQ. ID. No 5	*crtW*	β -carotene ketolase	SEQ. ID. No 6
SEQ. ID. No 7	*crtZ*	β -carotene hydroxylase	SEQ. ID. No 8

SEQ. ID. No 9	*crtY*	Licopene cyclase	SEQ. ID. No 10
SEQ. ID. No 11	*crtI*	Phytoene desaturase	SEQ. ID. No 12
SEQ. ID. No 13	*crtB*	Phytoene synthase	SEQ. ID. No 14
SEQ. ID. No 15	*crtE*	Geranylgeranyl pyrophosphate synthase	SEQ. ID. No 16

Genes provided by the present invention can be effectively used for the production of carotenoid by being inserted in various host cells. Those genes can be used either singly or together (more than 2, at least). For example, a gene coding licopene cyclase and represented by SEQ. ID. No 9 can be used for the production of β -carotene by being inserted in a microorganism containing *crtE*, *crtB* and *crtI* only. And, genes represented by SEQ. ID. No 5 and No 7, coding β -carotene ketolase and β -carotene hydroxylase respectively, can be used for the production of astaxanthine by being inserted in a microorganism producing β -carotene (ex: *Phaffia rhodozyma* ATCC96815).

The present invention further provides a carotenoid synthesis gene containing all the above genes.

A carotenoid synthesis gene is preferred to

have nucleotide sequences represented by SEQ. ID. No 4. The carotenoid synthesis gene (referred as '*crt* gene' hereinafter) of the present invention includes all the carotenoid synthesis genes involved in astaxanthine production process. An organization of *crt* gene of the present invention is presented in FIG. 15. As shown in FIG. 15, the size of *crt* gene of the present invention is 6,223 bp and includes *crtW*, *crtZ*, *crtY*, *crtI*, and *crtB* in that order in 5'→ 3' direction. Each of *Kpn* I, *Sma* I, *Xma* I, *Cla* I, *Hin*dIII and *Bam*H I recognition sequence is located therein. Stop codon of each *crtW*, *crtZ*, *crtY*, *crtI* and *crtB* is overlapped to start codon of the next gene. In particular, *crtE* gene is found as a complementary strand.

The present invention also provides a recombinant vector containing the above carotenoid biosynthesis gene.

A recombinant vector of the present invention was constructed by inserting *crt* gene into a basic vector. Any basic vector that was generally used for gene cloning or expression could be used for the present invention without limitation. And a choice of a vector depended on a host cell. For

example, if *E. coli* is used as a host cell, an *E. coli* specific vector having replication origin of the *E. coli* is preferred. Likewise, if yeast is used as a host cell, a yeast specific vector having replication origin of yeast is preferred. A shuttle vector that has both replication origin of *E. coli* and replication origin of yeast at the same time is also available. In the preferred embodiment of the present invention, the present inventors constructed a recombinant vector containing *crt* gene by using pCR-XL-TOPO vector, which was named 'pCR-XL-TOPO crtfull'.

The present invention also provides a strain prepared by transformed host cells with a recombinant vector containing the above carotenoid biosynthesis gene.

E. coli or yeast can be used as host cells of the present invention, and in particular, *E. coli* is preferably selected from a group consisting of XLI-Blue, TOPO, BL21(DE3) codon plus, DH1 and DH5α , but the choice is not always limited thereto. In a preferred embodiment of the present invention, a strain was prepared by transformed BL21(DE3) codon plus, a kind of *E. coli*, with a

recombinant vector 'pCR-XL-TOPO crtfull' which contained *crt* gene represented by SEQ. ID. No 4.

The present invention further provides a method for the production of carotenoid using the carotenoid biosynthesis gene.

The carotenoid producing method of the present invention is comprised of the following steps:

1) Cloning *crt* gene represented by SEQ. ID. No 4;

2) Constructing a recombinant vector in which the gene of the above step 1) was inserted;

3) Transforming a host cell with the recombinant vector of the step 2); and

4) Recovering carotenoids from the culture solution in which a strain transformed with the above recombinant vector was being cultured.

E. coli can be used as a host cell. At this time, any *E. coli* strain generally used for the transformation can be used without limitation, but it is preferred to select *E. coli* from a group consisting of XLI-Blue, TOPO, BL21(DE3) codon plus, DH1 and DH5α . In a preferred embodiment of the present invention, BL21(DE3) codon plus was

selected. The choice of a host cell for the invention is not limited to *E. coli*, and yeast is also available.

Particularly, a strain constructed in the above step 1) ~ step 3) was cultured in a growth medium (primary culture). Cells were recovered from the culture solution. Organic solvent was added to the cells, which was further cultured at 4℃ for overnight (secondary culture). At that time, it was possible to add IPTG (isopropyl-beta-D-thiogalactopyranside), an inducer inducing the production of carotenoid, into the culture solution. Carotenoid substrates such as FPP (farnesyl pyrophosphate), GGPP (geranylgeranyl diphosphate) or GPP (geranylpyrophosphate) could also be added. Methanol, acetone or ethyl ether could be used as an organic solvent for the extraction of carotenoid from the culture cells, and methanol was more preferred. Carotenoid was recovered from the culture cells by using HPLC (high performance liquid chromatography) or TLC (thin-layer chromatography) by following the conventional method commonly known for the people in this field.

In a preferred embodiment of the present invention, the inventors measured the amount of astaxanthine produced from a transgenic strain containing *crt* gene. As a result, the produced astaxanthine was 110 μg/g (dry weight), which was far more than that produced by an astaxanthine producing strain '*Paracoccus haeundaensis*' (25 μg/g (dry weight)). Therefore, the method for producing astaxanthine of the present invention makes possible even for a strain which cannot produce astaxanthine itself to mass-produce astaxanthine by using carotenoid biosynthesis gene, so that it facilitates the production of medical supplies and edible pigments as a food additive containing astaxanthine.

In an example of the present invention, a genomic DNA library of *Paracoccus haeundaensis* was constructed in order to clone a gene coding a protein required for carotenoid biosynthesis. The construction of a genomic DNA library was performed by the conventional method commonly known to the people in this field. In particular, a genomic DNA library was constructed by using a cosmid vector in this invention.

In another example of the present invention, 'color complementation' was used for cloning genes, involved in carotenoid biosynthesis, from a genomic DNA library. A microorganism not producing carotenoid (ex: *E. coli*) is given power to generate carotenoid by being transformed with a carotenoid biosynthesis gene that was cloned from a carotenoid producing microorganism (ex: *Paracoccus haeundaensis* of the present invention). For example, *E.coli* could produce β -carotene and cells turned into yellow after being transformed with *crtE, crtB, crtI* and *crtY*. And *E. coli* transformed with *crtE, crtB, crtI, crtY, crtW* and *crtZ* could produce astaxanthine and cells turned into orange. The present inventors cultured a genomic DNA library of *Paracoccus haeundaensis* in a medium supplemented with FPP (farnesyl pyrophosphate), a common substrate of carotenoid. As a result, 13 colonies having orange color were selected. Then, a cosmid vector was isolated from each colony. Nucleotide sequence of DNA insert included in the vector was identified. As a result, it was confirmed that the size of the smallest DNA insert was 6,223 bp. The nucleotide sequence of the DNA insert was represented by SEQ.

ID. No 4.

In another example of the present invention, nucleotide sequences of 6,223 bp long DNA insert assumed to contain a gene producing carotenoid was investigated, resulting in the obtainment of 6 ORFs. Those were analyzed nucleotided on NCBI GenBank. As a result, amino acid sequences translated from each ORF had a high homology with amino acid sequences of 6 enzymes involved in the reaction inducing astaxanthine production from FPP (see FIG. 9 - FIG. 14). From the result, the present inventors confirmed that the DNA insert isolated in this invention had *crt* gene coding a protein necessary for carotenoid biosynthesis (see FIG. 15).

In another example of the present invention, the inventors investigated if *E. coli* not producing carotenoid could produce it by the insertion of *crt* gene coding a relevant protein. First, recombinant vector 'pCR-XL-TOPO-crtfull', in which *crt* gene was inserted, was constructed (see FIG. 16). Then, the prepared recombinant vector was inserted in *E. coli*. Lastly, *E. coli* transformant having orange color was selected. In order to confirm if the transformant could produce

astaxanthine, the transformant was cultured and then cells were collected. Methanol was added to the obtained cells, which were cultured at 4℃ for overnight. Then, supernatant was obtained and optical density was measured at 190-900 nm. As a result, as shown in FIG. 17, original peaks of β-carotene and astaxanthine were confirmed. For more accurate analysis, HPLC assay was performed with some of the supernatant. At that time, β-carotene and astaxanthine purchased from Sigma were used as standard substances. As a result, it was confirmed that a transformant, in which *crt* gene isolated by the present inventors was inserted, could produce β-carotene and astaxanthine. The amount of astaxanthine produced from the transformant of the present invention was 110 μg/g (dry weight). The result suggested that *E. coli* in which *crt* gene was inserted could produce astaxanthine far more than *Paracoccus haeundaensis*, an astaxanthine producing strain, could do (25 μg/g (dry weight)).

BRIEF DESCRIPTION OF THE DRAWINGS

The application of the preferred embodiments

of the present invention is best understood with reference to the accompanying drawings, wherein:

FIG. 1 is a set of photographs showing the result of observation with a transmission electron microscope on the strain of the present invention in exponential stage, and each bar is 200 nm in length.

FIG. 2 is a graph showing the growth curve of the strain of the present invention depending on temperature.

FIG. 3 is a graph showing the growth curve of the strain of the present invention depending on pH.

FIG. 4 is a graph showing the growth curve of the strain of the present invention depending on culture time.

FIG. 5 is a graph showing the growth curve of the strain of the present invention depending on NaCl concentration.

FIG. 6 is a schematic diagram showing the phylogenetic dendrogram of the *Paracoccus* genus microorganisms of the present invention, based on 16S rDNA sequence data, and a bar represents maximum-parsimony distance (1 nucleotide substitutions per 100 nucleotides).

FIG. 7 is a set of graphs showing the results of scanning on the ranges of optical density changes at 190 ~ 890 nm (A) and at 400 ~ 550 nm (B) of a methanol extract extracted from the culture solution of the strain of the present invention. The peak at 450 nm is the peculiar peak of β -carotene, and the peak at 470 nm is the original peak of astaxanthine.

FIG. 8 is a schematic diagram showing the pathway of carotenoid biosynthesis.

FIG. 9 is a schematic diagram showing the result of comparison between an amino acid sequence of the first ORF (open reading frame) included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of β'-carotene ketolase (crtW) isolated from an

Alcaligenes genus microorganism and a *Bradyrhizobium* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Alcaligenes_sp: *Alcaligenes* genus microorganism

Bradyrhizobium_sp: *Bradyrhizobium* genus microorganism

Consensus: corresponding amino acid sequence

FIG. 10 is a schematic diagram showing the result of comparison between amino acid sequences of the second ORF included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of β-carotene hydroxylase (crtZ) isolated from an *Alcaligenes* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Alcaligenes_sp: *Alcaligenes* genus microorganism

Consensus: corresponding amino acid sequence

FIG. 11 is a schematic diagram showing the result of comparison between amino acid sequences of the third ORF included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of licopene cyclase (*crtY*) isolated from

an *Flavobacterium* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Flavobacterium_sp: *Flavobacterium* genus microorganism

Consensus: corresponding amino acid sequence

FIG. 12 is a schematic diagram showing the result of comparison between amino acid sequences of the fourth ORF included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of phytoene desaturase (*crtI*) isolated from an *Flavobacterium* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Flavobacterium_sp: *Flavobacterium* genus microorganism

Consensus: corresponding amino acid sequence

FIG. 13 is a schematic diagram showing the result of comparison between amino acid sequences of the fifth ORF included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of phytoene synthase (*crtB*) isolated from an *Flavobacterium* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Flavobacterium_sp: *Flavobacterium* genus microorganism

Consensus: corresponding amino acid sequence

FIG. 14 is a schematic diagram showing the result of comparison between amino acid sequences of the sixth ORF included in a DNA insert isolated from *Paracoccus haeundaensis* and amino acid sequences of geranylgeranyl pyrophosphate synthase (*crtE*) isolated from an *Flavobacterium* genus microorganism.

P. haeundaensis: *Paracoccus haeundaensis*

Flavobacterium_sp: *Flavobacterium* genus microorganism

FIG. 15 is a schematic diagram showing the organization of *crt* gene isolated from *Paracoccus haeundaensis*.

FIG. 16 is a schematic diagram showing the cleavage map of pCR-XL-TOPO-crtfull vector of the present invention.

FIG. 17 is a set of graphs showing the results of scanning on the ranges of optical

density changes at 190 ~ 890 nm (A) and at 350 ~ 550 nm (B) of a methanol extract extracted from the culture cells of the transformant of the present invention in which *crt* gene was inserted. The peak at 450 nm is the peculiar peak of β - carotene, and the peak at 470 nm is the original peak of astaxanthine.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Sample collection, microorganism separation, cultivation and maintenance of the same

Seawater sample taken from Haeundae shore in

Busan, Korea, was diluted by 1/1000, which was then smeared on a nutrient agar medium (Difco) and cultured at 25℃ for 3 days. Among microorganisms cultured thereby, those having orange or red color were isolated. In order to isolate a microorganism producing carotenoid among strains isolated, all the candidates were cultured in PPES-Ⅱ medium (tripton 1 g/ℓ , bacto-soyton 1 g/ℓ , ferric citrate 0.01 g/ℓ , polypeptone 2 g/ℓ and sodium chloride 3 g/ℓ). Cells were collected from the culture solution. Carotenoid was extracted by using methanol to identify a final strain producing carotenoid. The isolated microorganism was named 'BC74171^T strain'.

Example 2: Investigation of phenotypic characteristics

In order to investigate morphological and physiological characteristics of the BC74171^T strain of the present invention, experiments were performed as follows.

<2-1> Morphological characteristics

BC74171^T strain of the present invention was cultured in PPES-II medium at 25℃ for 3 days. The cultured cells were suspended in 0.1 M phosphate buffer (pH 7.2). The cells were fixed by 2% glutaraldehyde, washed with 0.05 M cacodylate buffer solution, and fixed again by 1% osmium tetroxide. The fixed cells were dehydrated in ethanol that was later replaced with propylene. Those cells were put into EPON resin and sections were prepared using an ultramicrotome. The prepared sections were observed under a JEM 1200EX-II transmission electron microscope (TEM).

As a result, as shown in FIG. 1, BC74171^T strain of the present invention had a shape of rod and was 0.3-0.7 ㎛ in diameter and 0.8-2.5 ㎛ in length. The strain did not form spores. The mobility of the strain was also observed under an optical microscope by hanging-drop technique (Skerman, V. B. D., *A Guide to the Identification of the Genera of Bacteria, 2nd den. Batimore,* 1967). As a result, BC74171^T strain of the present invention was non-motile. In addition, BC74171^T strain was Gram-negative, and its colonies were flat and had a light orange color.

<2-2> Physiological characteristics

1. Range of temperature

BC74171^T of the present invention was cultured in a nutrition agar medium (Difco) at different temperatures (4, 10, 20, 25, 28, 30, 37, 40 and 50℃) for 10 days to investigate the range of growth temperature. As a result, BC74171^T strain was growing at temperature ranging 20-37℃, and the optimum growth temperature was 25℃ (Table 2 and FIG. 2).

<Table 2>

Characteristics of *Paracoccus haeundaensis*

Morphological characteristics		Utilization	
Cell diameter(㎛)	0.3-0.7	D-Glucose	-
Cell length(㎛)	0.8-2.5	Maltose	-
Mobility	*-	D-Galactose	*+
Spore formation	-	Sucrose	-
Optimum growth temperature(℃)	25	Mannitol	-
Optimum growth pH	8	Cellobiose	-
NaCl resistance(%)	7	Trehalose	-

Product		Xylose	—
Indole	—	Dulcito	—
MR(Methyl red test)	—	Salicin	—
VP(Voges-Proskauer test)	—	Adonitol	—
Hydrogen sulfide	-	Inositol	—
Citrate	+	Arabinose	+
Enzyme activity		Raffinose	—
Catalase	+	Rhamnose	—
Urease	—	D-Fructose	—
Oxidase	+	D-Mannose	—
Starch hydrolysis	+	Dimethylformamide	—
Denitrification		Glycerol	—
Nitrate → Nitrite	+	L-Glutamic acid	—
Nitrite → N_2 gas	—	Sorbitol	—
Color	+(light orange)	Lactose	—
Chemotaxonomic characteristics		L-Asparagine	—
G + C content (mol%)	66.9	Acetone	—
Non-hydroxyl acids	$C_{18:1}$	Major carotenoid	Astaxanthine
3-Hydroxyl fatty acids	3-OH $C_{10:0}$		

*-: Positive response, *+: Negative response

2. Range of pH

BC74171^T of the present invention was cultured in PPES-II media each having different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0. 10.5 and 11.0) for 10 days to investigate growth pH range. As a result, the optimum growth pH was 8. The growth of the strain was inhibited or retarded under pH 6 and over pH 10.5 (Table 2 and FIG. 3).

3. Growth

BC74171^T strain of the present invention was shaking-cultured in a PPES-II medium at 25℃ for 10 days to investigate its growth by measuring turbidity. As a result, the growth of the strain was rapidly increased from 30 hours after the culture and slowly decreased from 50 hours after the culture (FIG. 4).

4. Resistance against NaCl

BC74171^T of the present invention was cultured in a trypticase soy broth medium containing 1-10%(w/v) NaCl for 10 days at various temperatures (4, 10, 20, 25, 28, 30, 37, 40 and 50℃). As a result, the optimum NaCl concentration for growth was 1-6%. The growth was retarded with 7% NaCl and was stopped over 8%

(Table 2 and FIG. 5).

5. Utilizing ability of carbon source

In order to examine a capacity to use a carbon source, a micro-plate including 24 substrates listed in the below Table 3 was used. The final concentration of each substrate was adjusted to 1%, which was loaded in a puple broth base (Difro), after being filtered or sterilized by moist heat. The medium was inoculated with the strain of the present invention, then was cultured at 25℃ for 10 days. Yellow meant a positive reaction and purple, which was an original color of the medium, meant a negative reaction.

<Table 3>

Substrates used in the carbon source utilizing ability test and the utilizing ability

	Substrate	Utilizing ability		Substrate	Utilizing ability
1	D-Glucose	*-	13	Arabinose	+
2	Maltose	-	14	Raffinose	-
3	D-Galactose	*+	15	Rhamnose	-
4	Sucrose	-	16	D-Fructose	-
5	Mannitol	-	17	D-Mannose	-

6	Cellobiose	-	18	Dimethylformamide	-
7	Trehalose	-	19	Glycerol	-
8	Xylose	-	20	L-Glutamic acid	-
9	Dulcitol	-	21	Sorbitol	-
10	Salicin	-	22	Lactose	-
11	Adonitol	-	23	L-Asparagine	-
12	Inositol	-	24	Acetone	-

*-: Not used (positive reaction),

+: Used (negative reaction)

As a result, as shown in Table 3, BC74171^T strain of the present invention used only D-arabinose and galactose as a carbon source, and no other substrates were used.

6. Activity of starch hydrolysis

In order to investigate if the strain of the present invention could hydrolyze starch, a starch agar was inoculated with the strain, then, cultured for 10 days. An activity of starch hydrolysis was measured by the method of Cowan, S. T. & Steel, K. J. (Cowan, S. T. & Steel, K. J., *Manual for the Identification of Medical Bacteria. London: Cambridge University Press,* 1965). As a

result, the strain of the present invention was confirmed to have an activity of starch hydrolysis (Table 2).

7. Indole test (tryptophanase activity)

In order to investigate if the strain of the present invention could produce indole by decomposing tryptophane, the present inventors took advantage of the method of Cappuccino *et al.* (Cappuccino J. G. and Sherman, N. In *Microbiology: a laboratory manual (6th)* 2001), resulting in the confirmation that indole was not produced from tryptophane (Table 2).

8. Acid production (fermentation) from carbohydrates

Acid production was investigated by the method of Hughm *et al.* (Hughm *et al.*, *J. Bacteriol.*, 66: 24-26, 1953). Methyl Red test and Voges-Proskauer test were performed and the results were all negative (Table 2).

9. Citric acid test

In order to investigate if the strain of the present invention could be growing by using citric

acid as a carbon source, the method of Cappuccino *et al.* (Cappuccino J. G. and Sherman, N. In *Microbiology: a laboratory manual (6th)* 2001) was performed and the result was positive (Table 2).

10. Catalase activity

The catalase activity was investigated by observing bubbles in 3% hydtogen peroxide solution by following the method of Harker *et al.* (Harker *et al., J. Clin. Microbiol.*, 2: 463-464, 1975). As a result, the catalse activity of the strain of the present invention was confirmed to be positive (Table 2).

11. Oxidase activity

The oxidase activity was investigated by using 1% p-aminodimethylaniline oxalate as a substrate by following the method of Cappuccino *et al.* (Cappuccino J. G. and Sherman, N. In *Microbiology: a laboratory manual (6th)* 2001). As a result, the cytochrome oxidase activity of the strain of the present invention was confirmed to be positive (Table 2).

12. Urease activity

The urease activity was measured by the method of Lanyi (Lanyi, B. *Methods Microbiol.*, 19: 1-67, 1987). As a result, the urease activity of the strain of the present invention was confirmed to be negative (Table 2).

13. Denitrification

In order to observe denitrification, gas production and growth were investigated through stab culture using a nutritive medium containing 0.1% (w/v) agar, by following the method of Cappuccino *et al.* (Cappuccino J. G. and Sherman, N. In *Microbiology: a laboratory manual (6th)* 2001). As a result, BC74171^T strain of the present invention reduced nitrate to nitrite. But, the strain could not reduce nitrite to N_2 gas (Table 2).

14. Hydrogen sulfide production test

In order to investigate if the strain of the present invention could produce hydrogen sulfide from amino acid containing sulfur like cysteine or a substrate like inorganic sulfur compounds, hydrogen sulfide production test was performed using TSI (Triple Sugar Iron Agar) medium

(Cappuccino J. G. and Sherman, N. In *Microbiology: a laboratory manual (6th)* 2001). The result was negative (Table 2).

Example 3: Fatty acid analysis

BC74171^T strain of the present invention was cultured on trypticase soy agar medium (pH 8.0) supplemented with 2% NaCl at 25℃ for 2 days. Then, harvesting, saponification, methylation and extraction of FAMEs (fatty acid methyl esters) were performed with the cells cultured by the method of Sasser (Sasser, M. *In Methods in Phytobacteriology*, 199-204, 1990). GC-MS was performed following the method of Lipske *et al.* (Lipski *et al.*, *Syst. Appl. Microbiol.*, 20: 448-457, 1997), leading to the investigation of FAMEs. As a result, according to the confirmed fatty acid profile ($C_{18:1}$, 84.32 %; $C_{18:0}$, 7.79 %; $C_{10:0}$ (3-OH), 2.06 %; C_{12}:1cis5 2.0 %; $C_{14:0}$ (3-OH), 1.47 %; $C_{17:0}$, 0.80 %; $C_{16:0}$, 0.78 %; and unknown peak, 0.78 %), the strain of the present invention was confirmed to belong to α-subclass of *Proteobacteria*. And a major hydroxyl fatty acid was $C_{10:0}$ (3-OH) (Table 2).

Example 4: Base composition of DNA

The base composition of genomic DNA of BC74171^T strain of the present invention was identified by the method of Tamaoka *et al.* (Tamaoka, J. & Komagata, K. *FEMS Microbiol. Lett.*, 25: 125-128, 1984). Particularly, the genomic DNA was extracted from BC74171^T strain of the present invention by following the conventional method known to the people in this field. The extracted genomic DNA was hydrolyzed. The obtained nucleotides were analyzed by HPLC (reverse-phase HPLC). As a result, G+C content of DNA of BC74171^T strain was 66.9 mol% (Table 2).

Example 5: 16S rDNA sequencing and phylogenetic dendrogram analysis

Genomic DNA was extracted from BC74171^T strain of the present invention by the method of Rainey *et al.* (Rainey *et al.*, *Syst. Appl. Microbiol.*, 15: 197-202, 1992). 16S rDNA was PCR amplified by using primers represented by SEQ. ID. No 1 and no 2. PCR was performed as follows.

pmol each of two primers, 10 ng of genomic DNA, 1 unit of Taq polymerase and 10x buffer solution□(with $MgCl_2$) were mixed to make the reacting solution. The prepared reacting solution was pre-denatured at 94℃ for 5 minutes, followed by 25 cycles of denaturation at 94℃ for 1 minute, annealing at 56℃ for 30 seconds, polymerization at 72℃ for 90 seconds, and final extension at 72℃ for 10 minutes. The PCR product was cloned into pGEM-T vector (Promega) and isolated DNA clones were reacted with ABI PRISM™ staining reagent (Perkin Elmer, USA). Nucleotide sequence was determined by using ABI 377 genetic analyzer (Perkin Elmer, USA).

As a result, 16S rDNA of BC74171^T strain of the present invention was 1451 bp long and had nucleotide sequences represented by SEQ. ID. No 3. The nucleotide sequence of 16S rDNA of BC74171^T strain was analyzed by using BLASTN and BLASTX of NCBI GenBank database. As a result, the nucleotide sequence of 16S rDNA of BC74171^T strain showed 99.8% and 99.6% homology each with those of *Paracoccus marcusii* and *Paracoccus carotinifaciens* which have been known to produce astaxanthine among various *Paracoccus* genus microorganisms.

Thus, BC74171^T strain of the present invention was confirmed to be a *Paracoccus* genus strain. And the characteristics of the strain of the present invention were compared with those of various *Paracoccus* genus microorganisms. The characteristics of other *Paracoccus* genus microorganism listed in the below Table 4 (2-15) were the results of investigations by Harker *et al.* (Harker *et al.*, *Int. J. Syst. Bacteriol.*, 48: 543-548, 1998), Lipski *et al.* (Lipski *et al.*, *Syst. Appl. Microbiol.*, 20: 448-457, 1997), Tsubokura *et al.* (Tsubokura *et al.*, *Int. J. Syst. Bacteriol.*, 49: 277-282, 1999), Kelly *et al.* (Kelly *et al.*, The genus *Paracoccus*. In *The Prokaryotes*, http://www.prokaryotes.com., Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer., 2000) and Doronina *et al.* (Doronina *et al.*, *Int. J. Syst. Evol. Bacteriol.*, 52: 679-6, 2002). As shown in Table 4, BC74171^T strain of the present invention did not share any characteristics with any other *Paracoccus* genus microorganism.

<Table 4>

Comparison of the characteristics of the strain of

the present invention and those of other *Paracoccus* genus microorganisms

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mobility		−	−	−	−	−	+	−	−	−	−	−	−	−	−	+
Growth in 6% NaCl		+	−	NR	−	−	NR	−	−	−	W	NR	NR	−	NR	NR
Usefulness	Glucose	−	+	−	+	+	+	+	−	+	+	+	+	−	+	+
	Arabinose	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−
	Glycerol	−	+	−	+	+	NR	+	−	+	+	+	+	−	−	+
	Sucrose	−	−	−	−	−	NR	+	−	−	+	+	+	−	−	+
	Mannitol	−	+	−	−	+	+	+	−	+	+	+	+	−	+	+
	Fructose	−	+	−	−	+	NR	+	−	+	+	+	+	−	+	+
Denitrification		−	−	+	−	−	−	+	+	+	−	−	+	+	+	+
Urease activity		−	+	NR	−	−	−	+	−	+	NR	+	−	NR	−	−
Yellow color		+	−	NR	−	−	+	−	−	−	+	−	−	−	−	−
G + C content (mol%)		66.9	64-66	NR	63	67	67	64-67	71	62.5	66	67	66	68.5-70	66.5-67.6	67-68

*1: The strain of the present invention

2: *P. alcaliphibus* (JCM 7364T)

3: *P. alkenifer* (DSM 11593T)

4: *P. aminophilus* (JCM 7686T)

5: *P. aminovorans* (JCM 7685T)

6: *P. carotinifaciens* (IFO 16121T)

7: *P. denitrificans* (ATCC 17741T)

8: *P. kocurii* (JCM 7684T)

9: *P. kondratievae* (VKM B-2222T)

10: *P. marcusii* (DSM 11574T)

11: *P. methylutens* (VKM B-2164T)

12: *P. pantotrophus* (ATCC 35512T)

13: *P. solventivorans* (DSM 6637T)

14: *P. thiocyanatus* (IAM 12816T)

15, *P. versutus* (ATCC 25364T)

*+: Positive reaction -: Negative reaction

W: Weak reaction) NR: Not reported

The characteristics of BC74171^T strain of the present invention were different in many ways even with *Paracoccus marcusii* and *Paracoccus carotinifaciens* showing the highest 16S rDNA sequence homology. More precisely, as shown in the below Table 5, *Paracoccus marcusii* (Harker *et al.*, *Int. J. Syst. Bacteriol.*, 48: 543-548, 1998) was a coccus or a short rod type forming a short chain. On the contrary, BC74171^T strain of the

present invention was a rod type not forming a chain. *Paracoccus carotinifaciens* (Tsubokura *et al.*, *Int. J. Syst. Bacteriol.*, 49: 277-282, 1999) was also a rod type not forming a chain but had flagella. However, BC74171^T strain of the present invention had not flagella. The strain of the present invention did not use any of glucose, mannitol, maltose or mannose, but the other two strains did use them. Besides, BC74171^T strain of the present invention decomposed starch. But, the two other strains did not.

<Table 5>

Comparison of the characteristics of the strain of the present invention and those of other *Paracoccus* genus microorganisms

Characteristics		BC74171^T	*Paracoccus marcusii*	*Paracoccus carotinifaciens*
Cell morphology		Rod type	Coccus or short rod type	Rod type
Mobility		—*	—	+*
Useful nesses	Glucose	—	+	+
	Maltose	—	+	+
	Mannitol	—	+	+
	Arabinose	+	+	—

Citric acid	+	+	−
Mannose	−	NR*	+
Starch hydrolysis	+	−	−
Color	+	+	+

*+: Positive reaction, −: Negative reaction, NR: Not reported

Considering all the above results, BC74171^T strain of the present invention was confirmed to be a novel microorganism belonging to *Paracoccus* genus. The present inventors named the BC74171^T strain as '*Paracoccus haeundaensis*' and deposited it at KCCM (Korean Culture Center of Microorganisms), on January 24, 2003 (Accession No: KCCM-10460). Systematic position of the *Paracoccus haeundaensis* of the present invention was shown in FIG. 6. Phylogenetic dendrogram was made out by using Treeview program. Bootstrap analysis (1000 replications) was performed using a method measuring distance and parsimony (Agnes Groisillier and Aline Lonvaud-Funel, *International Journal of Systematic Bacteriology*, 49: 1417-1428, 1999).

Example 6: Astaxanthine production in the strain of the present invention

The strain of the present invention was cultured in 50 ㎖ of medium (yeast extract 1%, tryptone 0.5%, NaCl 3%) at 25℃ for 6 days. The cells were collected by centrifugation (13,000 rpm). The collected cells were suspended in 20 ㎖ of methanol, which were then cultured overnight at 4℃. Centrifugation was performed again with 13,000 rpm to obtain supernatant. Optical density was measured at 190-900 nm.

As a result, as shown in FIG. 7, peaks were observed both at 450 nm and at 470 nm, and those peaks were confirmed to be original peaks of β-carotene and astaxanthine.

For the accuracy of analysis, 1 ㎖ of the supernatant was taken and filtered by a 0.45 ㎛ filter, followed by HPLC analysis (column: 4.6× 250 mm, uBondapak C18, Waters, Milford, MA; mobile phase: acetonitrile-methanol-water(49:44:7 v/v), Flow: 10 ㎖/min, Detector: 470 nm). β-carotene and astaxanthine purchased from Sigma were used as a standard substance. As a result, the strain of the present invention did produce astaxanthine and

β -carotene. The amount of astaxanthine produced from the strain was 25 μg/g (dry weight). Therefore, it was confirmed that bright orange color of the strain was caused by carotenoid biosynthesis in a cell and a major pigment accumulated in the cell was astaxanthine.

Example 7: Preparation of genomic DNA for the cloning of a carotenoid biosynthesis gene

Paracoccus haeundaensis (KCCM-10460) was cultured in a PPES-II medium (tripton 1 g/ℓ, bacto-soyton 1 g/ℓ, ferric citrate 0.01 g/ℓ, polypepton 2 g/ℓ and NaCl 3 g/ℓ) at 25℃ for 10 days. Then, the culture solution was centrifuged at 13,000 rpm to collect cells. In order to isolate genomic DNA from cells, the cells were suspended in STE buffer solution (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0), which was further reacted at 68℃ for 15 minutes. The cells obtained from centrifugation were re-suspended in solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0). 5 mg/mℓ of lysozyme and 100 μg/mℓ of RNase A were added, followed by reaction at 37℃ for 1 hour. Then, 250 μg/mℓ of proteinase K was added,

followed by reaction at 37℃for 3 hours. N-lauroylsarcosine was added by 1% of total volume, followed by reaction at 37℃. Genomic DNA was purified by phenol-chloroform extraction method. Extraction was performed by adding same volume of phenol-chloroform and genomic DNA was precipitated by adding 100% ethanol twice as much as the whole volume. The precipitated DNA was washed with 70% ethanol. TE buffer solution was added to dissolve the precipitated DNA completely at 65℃, which was then ready to be used.

Example 8: Construction of genomic DNA library

<8-1> Preparation of a cosmid vector

Xba I (9 U/㎍) restriction enzyme was added to 25 ㎍ of a cosmid vector (SuperCos 1 Cosmid Vector, Stratagene), making the total volume of reacting solution 200 ㎕, and the solution was reacted at 37℃ for 1 hour, followed by digestion. Vector DNA was purified by phenol-chloroform extraction method, which was precipitated by 100% ethanol. For dephosphorylation of the vector digested with *Xba* I, CIAP enzyme (Promega) was added, followed by reaction at 37℃ for 30 minutes.

Then, the purified vector was reacted with *Bam*H I (5 U/μg) at 37℃ for 1 hour. Extraction was performed by phenol-chloroform extraction method, and precipitation was induced by using ethanol. The precipitate was dissolved in TE buffer solution, making the concentration 1 μg/μℓ.

<8-2> Construction of genomic DNA library

100 μg of *Paracoccus haeundaensis* genomic DNA obtained in the above Example 7 was treated with Sau3A I (10 U), inducing a partial enzyme reaction. Upon completing the enzyme reaction, 0.5 M EDTA was added. Then, genomic DNA was separated by phenol-chloroform extraction method, which was precipitated by 100% ethanol. Genomic DNA, a product of a partial enzyme reaction, was dissolved in TE buffer solution, and treated with CIAP enzyme by the same method as used in the above Example 7, leading to dephosphorylation. DNA was separated again by phenol-chloroform extraction method. For the ligation with a cosmid vector prepared in the above Example <8-1>, T4 ligase (Promega) and 10x ligase buffer (Promega) were added to the isolated DNA, followed by reaction at 12℃ for 18 hours. After completing

the reaction, an *E. coli* strain XL1-Blue (Stratagene) was transformed with the ligation mixture, resulting in the construction of a genomic DNA library.

Example 9: Examination and analysis of a transgenic strain containing a color-producing gene

FPP (Sigma), one of common substrates included in carotenoids, was added to a LB agar medium by 1% out of total volume. A genomic library constructed in the above Example 8 was smeared on the plate, which was cultured at 37℃. Among cultured colonies, the ones having orange color were selected (13 out of about 2000 colonies). Cosmid vectors were isolated from those 13 colonies selected above. Then, primer working sequencing was performed to identify nucleotide sequences of DNA fragments inserted in each cosmid vector. Identification of the nucleotide sequences was committed to GenoTech Corp., Korea.

As a result, the smallest DNA insert of all fragments inserted in the cosmid vectors was 6,223 bp long, and had nucleotide sequences represented by SEQ. ID. No 4. A cosmid vector containing the nucleotide sequence represented by SEQ. ID. No 4 was named 'COSCRT'.

Example 10: Sequence analysis of a DNA insert containing a carotenoid biosynthesis related gene

Sequence analysis of a DNA insert obtained in the above Example 9 was performed using NCBI ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to analyze ORF.

As a result, 6 ORFs were included in a DNA insert obtained in the above Example 3. Each ORF showed a relevant homology with nucleotide sequences of *crtW* coding β-carotene ketolase, *crtZ* coding β-carotene hydroxlylase, *crtY* coding licopene cyclase, *crtI* coding phytoene desaturase, *crtB* coding phytoene synthase and *crtE* coding geranylgeranyl pyrophosphate synthase. All of those enzymes were confirmed to be involved in carotenoid biosynthesis.

The comparison of homology between amino acid sequences translated from each ORF and amino acid sequences of each carotenoid biosynthesis enzyme isolated from *Alcaligenes* sp. (Misawa *et al.*, *Biochem. Biophys. Res. Commun.*, 209(3): 867-876, 1995), *Bradyrhizobium* sp. (Hannibal *et al.*, *J. Bacteriol.*, 182(13): 3850-3853, 2000) and *Flavobacterium* sp. (Pasamotes *et al.*, *Gene*, 185(1): 35-41, 1997) was shown in FIG. 9 and FIG. 14. The nucleotide sequences of 6 ORF genes cloned by the present inventors and amino acid sequences translated therefrom were each represented by SEQ. ID. No 5 ~ No 16. In particular, *crtW*, *crtZ*, *crtY*, *crtI* and *crtB* genes were each represented by SEQ. ID. No 5, No 7, No 9, No 11, No 13 and No 15 and amino acid sequences of each gene above were represented by SEQ. ID. No 6, No 8, No 9, No 12, No 13 and No 16, respectively.

It was confirmed from the above results that *crt* gene involved in biosynthesis of carotenoid was included in a DNA fragment inserted in the cosmid vector above.

The composition of *crt* gene of the present invention was shown in FIG. 15. As shown in FIG. 15, termination codon and start codon were

overlapped in each *crtW*, *crtZ*, *crtY*, and *crtI*. Especially, *crtE* gene seemed to have a directionality of complementary strands, and had each of recognition sequences of *Kpn*I, *Xma*I, *Sma*I, *Cla*I, *Hind*III and *Bam*H in its sequences.

Example 11: Expression of *crt* gene in *E. coli*

In order to investigate if carotenoid could be produced by a protein expressed by *crt* gene of *Paracoccus haeundaensis* isolated in the above Example 9, *crt* gene was first amplified by PCR using HL premix (Bioneer). At that time, oligoneucleotide primers represented by SEQ. ID. No 17 and No 18 were used. All the PCRs were performed as follows; pre-denaturation at 94℃ for 5 minutes, denaturation at 94℃ for 30 seconds, annealing at 66℃ for 30 seconds, polymerization at 72℃ for 6 minute, 25 cycles from denaturation to polymerization, and final extension at 72℃ for 20 minutes. The PCR product was inserted in Topo-XL-vector (Invitrogen), which was used for transduction of *E. coli*. At that time, XL1-Blue (Stratagene), TOPO (Invitrogen), BL21(DE3) codon plus (Stratagene), DH1 (Takara) and DH5α (Takara)

were used as *E. coli* of the present invention. As a result, all transformants of BL21(DE3), XL1-Blue, BL21(DE3) codon plus were confirmed to have orange color. Thereafter, each transformant of *E. coli* was selected for culture. As a result, transformed BL21(DE3) codon plus produced astaxanthine most.

The present inventors inserted *crt* gene having nucleotide sequences represented by SEQ. ID. No 4, isolated in the above Example 9, into pCR-XL-TOPO vector (Invitrogen), a gene expression vector, which was then named 'pCR-XL-TOPO-crtfull' (FIG. 16). BL21(DE3) codon plus cells were transfected with the vector 'pCR-XL-TOPO-crtfull, followed by selection of strains having orange color. The selected strains were cultured in 50 ㎖ of LB medium at 37℃ for 8 hours. The culture solution was centrifuged at 13,000 rpm, then supernatant was discarded and cells were collected. 20 ㎖ of methanol was added to the collected cells. After vortexing, the cells were cultured at 4℃ for overnight. Centrifugation was performed (13,000 rpm) to obtain supernatant. In order to confirm whether carotenoid was generated, optical

density was measured at 190-900 nm and 400-550 nm. As a result, peaks were observed at 450 nm and 470 nm, which were confirmed to be original peaks of β -carotene and astaxanthine (FIG. 17).

For the accuracy of analysis, 1 ㎖ of the above supernatant was obtained and filtered with a 0.45 ㎛ (pore size) filter, followed by HPLC analysis (column: 4.6x250mm, uBondapak C18, Waters, Milford, MA; mobile phase: acetonitrile-methanol-water(49:44:7 v/v), Flow: 10ml/min, Detector: 470nm). At that time, β -carotene and astaxanthine, purchased from Sigma, were used as a standard substance. As a result, the substances produced from the strain of the present invention were confirmed to be astaxanthine and β -carotene.

Astaxanthine produced from the strain of the present invention was also quantified, resulting in the production of 110 ㎍/g (dry weight). For that result, neither inducer nor carotenoid substrate was added, suggesting that β -carotene and astaxanthine could be produced in *E. coli* only by nucleotide sequences having 6,223 bp isolated in the present invention. The amount of astaxanthine quantified above was far more than that produced (25 ㎍/g) by *Paracoccus haeundaensis*

(Accession No: KCCM-10460).

INDUSTRIAL APPLICABILITY

As explained hereinbefore, the present inventors have isolated and identified a novel *Paracoccus* genus microorganism mostly producing astaxanthine among many carotenoids, and also have cloned 6 genes coding proteins involved in carotenoid biosynthesis and *crt* gene containing the same from the above microorganism. The present inventors also have confirmed that carotenoid can be produced even in *E. coli* not producing carotenoid, by using *crt* gene. Therefore, a gene of the present invention and a microorganism producing the gene can be effectively used for the production of carotenoids such as β-carotene and astaxanthine which are available for making food, medicines and beauty stuffs.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily

utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.